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(54) Title: INHIBITION OF PROLIFERATION OF VASCULAR SMOOTH MUSCLE CELL

(57) Abstract

This invention encompasses a method for inhibiting vascular cellular activity of cells associated with vascular lesion formation in mammals which involves administering an effective dosage of at least one antisense sequence to at least one gene expressing a cyclin or a cyclin dependent kinase which inhibits the expression of the gene. More particularly, the invention involves administering antisense sequences which inhibit the expression of cyclin A, B1, B2, C, D1, D2, D3, E or cyclin X (p46) and cyclin dependent kinase cdc2, cdk2, cdk4 or cdk5. It is preferable to use two antisense sequences each from a different cyclin or cyclin dependent kinase. The cyclin or cyclin depending kinase dosage is preferably administered in combination with proliferating cell nuclear antigen (PCNA). Antisense methods and compositions direct to inhibiting the expression of growth factors such as TGF-β1, TGF, bFGF, PDGF are also contemplated by the present invention. The antisense sequences are incorporated into liposomes, particularly liposomes containing HVJ and which are directly administered intraluminally, intramurally or periadventitiously. While the methods of this invention are useful in treating a broad spectrum of vascular lesions such as lesions in the carotid femural and renal arteries, particularly lesions resulting from renal dialysis fistulas. The invention is particularly useful in treating vascular lesions associated with coronary artery angioplasty.

INHIBITION OF PROLIFERATION OF
VASCULAR SMOOTH MUSCLE CELL

Reference to related applications

5   This is a continuation-in-part of U.S.S.N. 08/063,980 filed May 19, 1993, which in turn is a continuation-in-part of U.S.S.N. 07/944,882 filed September 10, 1992, the contents of both which are incorporated herein by reference.

10

BACKGROUND OF THE INVENTION

Technical Field

The field of this invention is inhibition of cellular activity related to vascular lesions.

5

Background of The Related Art

In the growth and maintenance of multi-cellular organisms, the organism has had to develop processes to activate or inhibit the proliferation of cells. The organism has developed numerous mechanisms, whereby signals are given to cells, by either

10 intracellular or extracellular messengers. Control of proliferation provides for modeling of organs, maintenance of subsets of leukocytes in hematopoiesis, wound healing and the like. However, in many situations, such as injury or disease states, the response of the organism to the injury or disease may, in fact, be deleterious to the health of the organism.

15 The vascular response to injury involves an alteration in three fundamental cellular processes; cell growth, cell migration and extracellular matrix production. This vascular response to injury is characteristic of the pathogenesis of various vascular diseases including (but not limited to): atherosclerosis, restenosis after angioplasty, vein bypass graft stenosis, prosthetic graft stenosis, angiogenesis and hypertension. For

20 example, atherosclerotic lesions evolve as a result of vascular smooth muscle migration into the subintimal space, proliferation and the production of abundant extracellular matrix. Similarly, restenosis after angioplasty, vein bypass graft stenosis, prosthetic graft stenosis, angiogenesis and hypertension involve abnormalities in vascular cell growth, migration and matrix composition. The precise mechanisms responsible for

25 alterations in the regulation of these cellular processes are poorly characterized.

Dzau, Hypertension 8, 553-559 (1986) describes the vascular renin-angiotensin pathway. Geisterfer, et al., Circulation Research 62, 749-756 (1988) report that angiotensin II induces hypertrophy of cultured rat aortic smooth muscle cells. Barrett and Benditt, Proc. Natl. Acad. Sci. USA 85, 2810-2814 (1988) describe the expression

30 of platelet-derived growth factor in human atherosclerotic plaques and normal artery

wall. Powell, et al., <u>Science</u> 245, 186-188 (1989) report that inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. Naftilan, et al., <u>J. Clin. Invest.</u> 83, 1413-1424 (1989) report the induction of platelet-derived growth factor A chain and c-myc gene expression by angiotensin II in cultured rat vascular

5   smooth muscle cells. Sarzani, et al., <u>J. Clin. Invest.</u> 83, 1404-1408 (1989) describe the expression of various growth factors in aorta or normotensive and hypertensive rats. Majesky, et al., <u>J. Clin. Invest.</u>, 88, 904-910 (1991) report the production of TGF-β_1 during repair of arterial injury. Linder, et al., <u>Circulation Research</u>, 68, 106-113 (1991) describe the role of basic fibroblast growth factor in vascular lesion formation.

10   Daemen, et al., <u>Circulation Research</u>, 68, 450-456 (1991) report the role of angiotensin II in inducing smooth muscle cell proliferation in the normal and injured rat arterial wall. Ferns, et al., <u>Science</u>, 253, 1129-1132 (1991) report the inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF, Gibbons, et al., <u>Clin. Research</u>, 38, 287A (1990) report the modulation by transforming growth factor-β

15   of the bifunctional growth response of vascular smooth muscle cells to angiotensin II. Itoh, et al., <u>Biochem. Biophys. Res. Comm.</u>, 176, 1601-1609 (1991) report the interaction of atrial natriuretic polypeptide and angiotensin II on protooncogene expression and vascular cell growth.

Simons and Rosenberg, <u>Circ. Res.</u>, 70, 835-843 (1992) report that antisense

20   oligonucleotides to non-muscle myosin heavy chain and c-myb suppress smooth muscle cell proliferation *in vitro*. Speir and Epstein, <u>Circulation</u>, 86, 538-547 (1992) report that antisense to proliferating cell nuclear antigen (PCNA) inhibits smooth muscle cell proliferation *in vitro*. Rosenberg et al., PCT/US92/05305, January 1993 describes an antisense approach of localized oligonucleotide therapy involving the inhibition of the

25   c-myb or PCNA protein.

The relationship of the expression of cyclins at various phases of the cell synthesis and mitosis cycle are described in <u>The Journal of NIH Research</u>, December 1992, Vol. 4, pp. 55-59. Figure 1 for example shows the approximate level of Cyclins A, B1, B2, C, D1, D2 and E during G1 (gap 1), synthesis, G2 (gap 2) and mitotic phase

of cell proliferation. Williams, et al., _The Journal of Biological Chemistry_, Vol. 266, No. 12, April 25 (1993), pp. 8871-8880 sh ws the isolation f cyclin x (p46).

Both of these later two articles and references such as O'Connor, et al., _The Journal of Biological Chemistry_, Vol. 286, No. 11, April 15, pp. 8298-8308 (1993)

5   elucidate the interaction of various Cyclin dependent kinases such as cdc2 and cdk2 and Cyclins A and B.

Jaskulski et al., _Science_, Vol. 240, No. 4858, pp. 1544-6 (1988) describes the effects of antisense oligonucleotides on growing Balb/C313 cells. It was shown that the antisense molecule inhibited synthesis and mitosis and sense oligos had no effect.

10   Sala et al., _Proc. Natl. Acad. Sci., USA_, Vol. 89, No. 21, pp. 10415-9 (Nov. 1992) describes antisense sequences to c-myb gene which inhibit c-myb expression. Kimeki, _J. Cell Biochem_, Vol. 50, No. 1, pp. 1-9 (1992) describes antisense interruption of C-myc or Cyclin A _in vitro_. Doyle et al., _Antisense Res. Dev._, Vol. 1, No. 1 Spring, pp. 11-20 (1991) describes antisense oligonucleotides to Cyclin B in xenopus oocytes.

15   Zindly et al., _Biochem Biophys. Res. Commun._, Vol. 1897, No. 3, pp. 1144-54 (1992) describes antisense interruption of the production of Cyclin A or Cyclin B in rats. Cyclin A antisense oligonucleotides are also described in _Nature_, Vol. 354, No. 6351, pp. 314-7 (1991) and Guerria et al., _EMBO J._, Vol. 10, No. 11, pp. 3343-9 (1991). These references report the effect of Cyclin A or Cyclin B oligos in metaphase I

20   oocytes. Further, antisense studies in xenopus embryos are described in _Development_, Vol. III, No. 4 pp. 1173-8 (1991) and _J. Cell. Biol._, Vol. 114, No. 4, pp. 767-72 (1991). Lapidot-Lifson et al., _Proc. Natl. Acad. Sci. USA_, Vol. 89, No. 2, pp. 579-83 (1992) describe the down regulation of cdc2 with antisense oligonucleotides.

SUMMARY OF THE INVENTION

This invention encompasses a method for inhibiting vascular cellular activity of cells associated with vascular lesion formation in mammals which involves administering an effective dosage of at least one antisense sequence to at least one mRNA or pre-
5   LRNA for a gene for a cyclin or a cyclin dependent kinase which antisense sequence inhibits the expression of the gene.   More particularly, the invention involves administering antisense sequences which inhibit the expression of cyclin A, B1, B2, C, D1, D2, D3, E or cyclin X (p46) and cyclin dependent kinase cdc2, cdk2, cdk4 or cdk5. It is preferable to use two antisense sequences each which inhibits expression of
10 a different cyclin or cyclin dependent kinase.

In another aspect, the antisense sequence against cyclin or cyclin dependent kinase is preferably administered in combination with an antisense sequence against proliferating cell nuclear antigen (PCNA).

Antisense methods and compositions directed to inhibiting the expression of
15 growth factors such as transforming growth factor beta (TGFβ), basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) are also contemplated by the present invention.

The antisense sequences are incorporated into liposomes, particularly liposomes containing HVJ and which are directly administered intraluminally, intramurally or
20 periadventitiously.

While the methods of this invention are useful in treating a broad spectrum of vascular lesions. Such lesions include, but are not limited to, lesions in the carotid femoral and renal arteries, particularly lesions resulting from renal dialysis fistulas. The methods of the present invention are particularly useful in treating vascular lesions
25 associated with cardiovascular angioplasty. For such use, the antisense sequence is applied to the angioplasty site to reduce restenosis.

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DETAILED DESCRIPTION OF THE INVENTION

Brief Description of the Drawings

	Figure 1	is a schematic diagram of the appropriate level of various cyclins in G1, S, G2, or M phase states of the cell cycle
5	Figure 2	shows the histology of restenosis
	Figure 3	shows the effect of combined antisense treatment against cdc2 and PCNA on neointima with time
	Figure 4	shows the effect of antisense against cdk2 and cdc2 on neointimal/media areas ratio.
10	Figure 5	long term efficiency of antisense and sense ODN of cdc2 kinase/PCN on intimal/medial area ratio. **$P < 0.01$ vs. sense.

The approximate levels of expression of cyclins during the cell cycle are shown in Figure 1, Cyclin D, appears in the gap 1 (G1) phase and is expressed throughout the cycle in the presence of growth factors. Cyclin C accumulates in early G1 and then

15 declines. Cyclin D2 and E accumulate at the end of G1 and are degraded during synthesis (S) phase Cyclin A is first detected in the (S) phase and is degraded as the cells enter mitosis (M). Cyclins B1 and B2 appear soon after cyclin A and are degraded in mid-M phase. These cyclins are important proteins in the cell cycle and are recognized as a family of related proteins by their amino acid sequence homology.

20 Cyclins form active complexes with kinases and these active complexes phosphorylate proteins.

Figure 2 illustrates the restenosis process after angioplasty. Angioplasty is performed by inflating a balloon in an artery to relieve an obstructive atherosclerotic lesion. In the course of compressing the lesion the procedure induces tearing of the

25 internal elastic laminae (IEL). Increased blood flow results from relief of the narrowing. The tears in the IEL and injury of the blood vessel wall by the balloon expansion induce a vascular smooth muscle cell proliferative response that results in formation of a neointima and renewed obstruction to the vessel lumen. The neointima consists of smooth muscle cells and matrix and takes 1-6 months to form post

30 angioplasty.

Tables 1 and 2 illustrate sense and antisense sequences for various growth factors, for representative cyclins or, cyclin dependent kineses, and PCNA. Table 3 illustrates various human DNA sequences useful for deriving antisense sequence information for making therapeutic agents of this invention. Table 4 illustrates the sequence of ODN against cell regulatory genes used in various examples. Table 5 are antisense ODN on neointima/medial area ratios as determined in Example 4.

TABLE 1

Genbank Accession #

A.   human TGF-β_1 MRNA: Nature 316,701-706(1985),

X02812

5   J05114

5'-GCC UCC CCC AUG CCG CCC UCC GGG-3'[SEQ ID NO:1]

antisense TGF:

3'-GGG TAC GGC GGG AGG-5' [SEQ ID NO:2]

control TGF:

10   sense TGF:

5'-CCC ATG CCG CCC TCC-3 [SEQ ID NO:3]

reverse TGF:

5'-GGG TAC GGC GGG AGG-3'[SEQ ID NO:4]

B.   human BFGF MRNA: N.Y. Acad.Sci. 638, 109-123 (1991)

15   Biochem, Biophys. Res. Commun. 144 543-550(1988)

(GCA) 5'-GCA GGG ACC AUG GCA GCC GGG AGC-3' [SEQ ID NO:5]

M17599

S81809

antisense FGF:

20   3'-CCC TGG TAC CGT CGG-5' [SEQ ID NO:6]

control FGF:

sense FGF:

5'-GGG ACC ATG GCA GCC-3' [SEQ ID NO:7]

reverse FGF:

25   5'-CCC TGG TAC CGT CGG-3' [SEQ ID NO:8]

C.   human PDGF A chain mRNA:Nature 320, 695-699(1986)

5'-CGG GAC GCG AUG AGG ACC UUG GCU-3' [SEQ ID NO:9] X03795

antisense PDGF:

3'-TAC TCC TGG AAC CGA-5' [SEQ ID NO:10]

30   control PDGF:

sense PDGF:

   5'-ATG AGG ACC TTG GCT-3' [SEQ ID NO:11]

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TABLE 2

A.   Mouse cdc2 Kinase mRNA   <u>Genbank Accession #</u>

      5'-UGA GUA ACU AUG GAA GAC UAU AUC-3' [SEQ ID NO:12]X16461

5   Antisense

           3'-ACT CAT TGA TAC CTT CTG-5' [SEQ ID NO:13]

     Sense

           5'-TGA GTA ACT ATG GAA GAC-3' [SEQ ID NO:14]

B.   Rat PCNA mRNA   Y00047 and M24604

10   5'-AAC UCC GCC ACC AUG UUU GAG GCA GGC CUG-3' [SEQ ID

NO:15]

     Antisense

           1:       -6 +9

                3'-CGG TGG TAC AAA CTC-5' [SEQ ID NO:16]

15   2:   +4- +21

                3'-AAA CTC CGT GCG GAC TAG-5' [SEQ ID NO:17]

     Sense

           1:       -6 +9

                5'-GCC ACC ATG TTT GAG-3' [SEQ ID NO:18]

20   2:   +4- +21

                5'-TTT GAG GCA CGC CTG ATC-3' [SEQ ID NO:19]

C.   Rat cyclin B mRNA   X60768

                5'-GGA GCC AUG GCG CUC AGG GUC-3' [SEQ ID NO:20]

     Antisense Cyclin B

25   3'-CCT CGG TAC CGC GAG TCC-5' [SEQ ID NO:21]

     Sense Cyclin B

           5'-GGA GCC ATG GCG CTC AGG-3' [SEQ ID NO:22]

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TABLE 3

A.   Human cdk2 Kinase mRNA, EMBO J 10 2653-2659 (1990)

X61622

5   5'-UGG CGC UUC AUG GAG AAC UUC CAA-3' [SEQ ID NO:23]

Antisense cdk2:

3'-GCG AAG TAC CTC TTG AAG-5' [SEQ ID NO:24]

Control

Sense cdk2

10   5'-CGC TTC ATG GAG AAC TTC-3' [SEQ ID NO:25]

B.   Human cdc2, Nature 327:31-35 (1987)   X05360

5'-TTGACTAACTATGGAAGATTATACCAAAATAGAGAAAATT

GGAGAAGGTACCTATGGAGTTGTGTATAAGGGT-3' [SEQ ID NO:26]

C.   Human cyclin A Nature 343:555-557 (1990)   X51688

15   5'-GGGAGCAGTGATGTTGGGGCAACTCTGCGCCGGGGCCTGCG   [SEQ ID
NO:27]

D.   Human cyclin B Cell 58:833-846 (1989)   M25753

5'-AGAGGAAGCCATGGCGCTCCGAGTCACCAGGAACTCGAAA

ATTAATGCTGAAAATAAGGCGAAGATCAACATG-3' [SEQ NO:28]

20   E.   Human cyclin C Cell 66:1197-1206 (1991)   M74091

5'-GGCTGGGTCTATGGTCGCTCCGCGGCCGTCCGCCGCGTGG

TGCTTTTTTATCAGGGCAAGCTGTGTTCCATGGCAGGGAAC-3'[SEQIDNO:29]

F.   Human cyclin D1 Cell 66:1197-1206 (1991)   M74092

5'-AGCCCCAGCCATGGAACACCAGCTCCTGTGCTGCGAAGTGG

25   AAACCATCCGCCGCGCGCTACCCCGATGCCAACCTCCTCAAC-3'   [SEQ ID
NO:30]

G.   Human cyclin D3 J. Biol.Chem. 267:20412-20415

(1992)   M92287

5'-CTGCCCGAGTATGGAGCTGCTGTGTTGCGAAGGCACCCGGC

30   ACGCGCCCCGGGCCGGGCCGGACCCGCGGCTGCTGGG-3' [SEQ ID NO:31]

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H.   Human cyclin E <u>Cell</u> 66:1197-1206 (1991)   M74093

5'-GCGGGACACCATGAAGGAGGACGGCGGCGCGGAGTTCTCGG

CTCGCTCCAGGAAGAGGAAGGCAAACGTGACCGTTTTTTG-3' [SEQ ID NO:32]

I.   Human PCNA <u>J. Biol. Chem.</u> 264:7466-72 (1989)   J04718

5   5'-CTCCGCCACCATGTTCGAGGCGCGCCTGGTCCAGGGCTCCA

TCCTCAAGAAGGTGTTGGAGGCACTCAAGGACCTC-3' [SEQ ID NO:33]

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TABLE 4

Table 4. Sequences of Antisense ODN.

1) cdc 2 kinase (position -9 to +9 of the mouse sequence)

Sense   5'-TGA-GTA-ACT-ATG-GAA-GAC-3' [SEQ ID NO:34]

Antisense   5'-GTC-TTC-CAT-AGT-TAC-TCA-3' [SEQ ID NO:35]

2) cdc 2 kinase (position -9 to +9 of the rat sequence)

Sense 5'-TGA-GTA-ACT-ATG-GAG-GAC-3' [SEQ ID NO:36]

Antisense   5'-GTC-TTC-CAT-AGT-TAC-TCA-3' [SEQ ID NO:37]

3) PCNA (position +4 to +22 of the rat sequence)

Sense 5'-TTT-GAG-GCA-CGC-CTG-ATC-3' [SEQ ID NO:38]

Antisense   5'-GAT-CAG-GCG-TGC-CTC-AAA-3' [SEQ ID NO:39]

4) cdk 2 kinase (position -6 to +12 of human sequence)

Sense 5'-CGC-TTC-ATG-GCG-AAC-TTC-3' [SEQ ID NO:40]

Antisense   5'-GAA-GTT-CTC-CAT-GAA-GCG-3' [SEQ ID NO:41]

5) cyclin B1 (position of -9 to +9 of rat sequence)

Sense 5'-GAA-GGA-GCC-ATG-GCG-CTC-3' [SEQ ID NO:42]

Antisense   5'-GAG-CGC-CAT-GGC-TCC-TCC-3' [SEQ ID NO:43]

6) Scrambled ODN

5'-CTT-CGT-CGG-TAC-CGT-CTT-C-3' [SEQ ID NO:44]

7) thrombomodulin

Antisense   5'-ACC-CAG-AAA-GAA-AAT-CCC-3' [SEQ ID NO:45]

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Table 5. Effect of antisense ODN on neointima/medial areas ratio

	SENSE	ANTISENSE	UNTRANSFECTED
PCNA	n.t.	1.460 ± 0.193	n.t.
cdc3 kinase			
mouse	1.250 ± 0.188	$0.741 \pm 0.126*$	1.244 ± 0.253
rat	n.t.	$0.814 \pm 0.201**$	1.856 ± 0.208
cdk 2 kinase	1.341 ± 0.307	$0.597 \pm 0.131**$	1.156 ± 0.077
cdc 2 (mouse) + PCNA	1.174 ± 0.235	$0.084 \pm 0.06**$	1.510 ± 0.203
cdc 2 (rat) + PCNA	n.t.	$0.130 \pm 0.074**$	0.931 ± 0.27
cdc 2 (rat) + cyclin B1	n.t.	$0.267 \pm 0.029**$	1.220 ± 0.189
cdc 2 (mouse) + cdk 2	1.440 ± 0.366	$0.283 \pm 0.188**$	n.t.
scrambled	1.695 ± 0.136		
thrombomodulin	n.t.	1.551 ± 0.099	1.625 ± 0.183
injured (vehicle)	1.759 ± 0.398		
injured (untreated)	1.432 ± 0.188		

* $P < 0.05$, ** $P < 0.01$ vs. sense treated and/or untransfected vessels.
n.t. = not tested, vehicle = treated with HVJ complex without ODN, N = 4-8 rats.

The invention is related to a method for inhibiting expression of protein-encoding genes using antisense oligonucleotides. The method is based on the localized application of the oligonucleotides to a specific site *in vivo*. The oligonucleotides preferably are applied directly to the target tissue in a mixture with an implant or gel, or by direct

5   injection or infusion. In one aspect, the oligonucleotides are modified to render them resistant *in vivo* to degradation or alteration by endogenous enzymes.

The therapeutic approach using antisense oligonucleotides is based on the principle that the expression of a protein from a gene can be down regulated by providing an appropriate length oligonucleotide which is complementary to at least a

10   portion of the messenger RNA (MRNA) transcribed from the gene. The antisense strand hybridizes with the mRNA and targets the MRNA for down regulation thereby preventing ribosomal translation, and subsequent protein synthesis.

Preferred Oligomers

15   The Oligomer selected may be any of a number of types, including those having a charged or uncharged backbone.

Preferred Oligomers include alkyl- and aryl- phosphorate Oligomers, especially preferred are methylphosphonate Oligomers. Other preferred Oligomers include phosphorothioate Oligomers, phosphorodithioate Oligomers, morpholino analogs,

20   formacetal analogs, thioformacetal analogs and peptide nucleic acid ("PNA") analogs.

Preferably the Oligomers each comprise from abut 4 to about 40 nucleosides, more preferably, from about 6 to 30 nucleosides. Especially preferred are Oligomers of about 8 to about 20 nucleosides.

Preferred target regions when the nucleic acid target sequence is mRNA or pre-mRNA include the 3'-untranslated region, the coding region, splice sites of pre-mRNA (including splice donor and splice acceptor sites), the initiation codon region including regions slightly downstream of the AUG start codon (preferably up to about 20

5   nucleotides downstream from the AUG initiation codon), regions immediately 5'- of the initiation codon (preferably about 5 to 200 nucleotides 5'- of the AUG start codon) and the 5'-cap cite.

According to an alternately preferred aspect, tandem Oligomers are employed. Preferred tandem Oligomers include those which comprise a total of about 20 to about

10   40 nucleosides.

Oligomers having the selected internucleoside linkages may be conveniently prepared according to synthetic techniques known to those skilled in the art. For example, commercial machines, reagents and protocols are available for the synthesis of Oligomers having phosphodiester and certain other phosphorus-containing

15   internucleoside linkages. See also Gait, M.J., Oligonucleotide Synthesis: A Practical Approach (IRL Press, 1984); Cohen, Jack S., Oligodeoxynucleotides Antisense Inhibitors of Gene Expression, (CRC Press, Boca Raton, FL, 1989); Oligonucleotides and Analogues: A Practical Approach, (F. Eckstein, 1991); and Agarwal, S., Protocols for Oligonucleotides and Analogs Synthesis and Properties, (Humana Press, N.J., 1993).

20   Preparation of Oligomers having certain non-phosphorus-containing internucleoside linkages is described in United States Patent No. 5,142,047, the disclosure of which is incorporated herein by reference.

Synthetic methods for preparing methylphosphonate Oligomers are described in Lee B.L., et al., Biochemistry 27:3197-3203 (1988), Miller, P.S., et al., Biochemistry 25:5092-5097 (1986), and "An Improved Method for the Synthesis and Deprotection of Methylphosphonate Oligonucleotides" in Protocols for Oligonucleotides and Analogs

5   Synthesis and Properties, S. Agarwal, ed. (Humana Press, N.J. 1993); and published PCT applications WO 92/07864 and WO 92/07882 the disclosures of which are incorporated herein by reference.

Also preferred are Oligomers which are nucleoside/non-nucleoside polymers. Suitable Oligomers also include chimeric oligonucleotides which are composite RNA,

10   DNA analogues (Inouo et al., FEBS Lett. 2115:327 (1987)). Other suitable Oligomers include Oligomers having chimeric backbones. Such chimeric backbone Oligomers include Oligomers having mixed phosphate backbones including nucleoside sequences which are capable of activating RNaseH, nucleoside sequences which do not activate RNaseH, and thus allow site directed cleavage of an RNA molecule. See U.S. Patent

15 · No. 4,149,797 which is incorporated herein by reference. Chimeric backbone Oligomers also include Oligomers having a mixture of internucleosidyl linkages which may or may not include phosphorus atoms, such as morpholinyl linkages, formacetal linkages, peptide nucleic acid (PNA) linkages and the like. Oligomers having a neutral backbone, for example, methylphosphonate Oligomers with cleaving or cross-linking

20 · moieties attached, may prove advantageous in certain circumstances; such Oligomers may have a longer half-life in vivo since the neutral structure reduces the rate of nuclease digestion while the cleaving or cross-linking moiety may promote inactivation of target polynucleotide sequences.

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According to one aspect of the present invention, these antisense Oligomers have a sequence which is complementary to a portion of the RNA transcribed from the selected target gene. Although the exact molecular mechanism of inhibition has not been conclusively determined, it has been suggested to result from formation of duplexes

5   between antisense Oligomer and the RNA transcribed from the target gene. The duplexes as formed may inhibit translation, processing or transport of an mRNA sequence or may lead to digestion by the enzyme RNaseH.

Single stranded Oligomers may also bind to a duplex DNA target such that a duplex is formed with one of the two DNA strands, and the second DNA of the target

10   strand is displaced from the duplex. Preferred is the formation of a duplex by the Oligomer with the coding strand of the DNA duplex target ("invading duplex"). The invading duplex so formed may inhibit transcription.

As a general matter, the Oligomer employed will have a sequence that is complementary to the sequence of the target nucleic acid. However, absolute

15   complementarily may not be required; in general, any Oligomer having sufficient complementarily to form a stable duplex with the target nucleic acid is considered to be suitable. Since stable duplex formation depends on the sequence and length of the hybridizing Oligomer and the degree of complementarily between the antisense Oligomer and the target sequence, the system can tolerate less fidelity (complementarily) when

20   longer Oligomers are used. However, Oligomers of about 8 to abut 40 nucleosidyl units in length which have sufficient complementarily to form a duplex having a melting temperature of greater than about 40°C under physiological conditions are particularly suitable for use according to the method of the present invention.

Definitions

As used herein, the following terms have the following meanings unless expressly stated to the contrary.

The term "purine" or "purine base" includes not only the naturally occurring

5   adenine and guanine bases, but also modifications of those bases such as bases substituted at the 8-position, or guanine analogs modified at the 6-position or the analog of adenine, 2-amino purine, as well as analogs of purines having carbon replacing nitrogen at the 9-position such as the 9-deaza purine derivatives and other purine analogs.

10   The term "nucleoside" includes a nucleosidyl unit and is used interchangeably therewith, and refers to a subunit of a nucleic acid which comprises a 5-carbon sugar and a nitrogen-containing base. The term includes not only those nucleosidyl units having A, G, C, T and U as their bases, but also analogs and modified forms of the naturally-occurring bases. In RNA, the 5-carbon sugar is ribose; in DNA, it is 2'-

15   deoxyribose. The term nucleoside also includes other analogs of such subunits, including those which have modified sugars such as 2'-O-alkyl ribose.

20   The term "phosphorate" refers to the group $\begin{array}{c} | \\ O \\ | \\ O{=}P{-}R \\ | \\ O \\ | \end{array}$
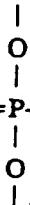
25

Wherein R is hydrogen or an alkyl or aryl group. Suitable alkyl or aryl groups include those which do not sterically hinder the phosphorate linkage or interact with each other.

- 20 -

The phosphorate group may exist in either an "R" or an "S" configuration. Phosphorate groups may be used as internucleosidyl phosphorus group linkages (or links) to connect nucleosidyl units.

5

The term "phosphodiester" or "diester" refers to the group $O=P-O$ with additional O substituents above and below

$$\begin{array}{c} | \\ O \\ | \\ O=P-O \\ | \\ O \\ | \end{array}$$

10

wherein phosphodiester groups may be used as internucleosidyl phosphorus group linkages (or links) to connect nucleosidyl units.

A "non-nucleoside monomeric unit" refers to a monomeric unit wherein the base, the sugar and/or the phosphorus backbone has been replaced by other chemical moieties.

A "nucleoside/non-nucleoside polymer" refers to a polymer comprised of nucleoside and non-nucleoside monomeric units.

The term "oligonucleotide" or "Oligomer" refers to a chain of nucleosides which are linked by internucleoside linkages which is generally from about 4 to about 100 nucleosides in length, but which may be greater than about 100 nucleosides in length. They are usually synthesized from nucleoside monomers, but may also be obtained by enzymatic means. Thus, the term "Oligomer" refers to a chain of oligonucleosides which have internucleosidyl linkages linking the nucleoside monomers and, thus, includes oligonucleotides, nonionic oligonucleotide alkyl-and aryl-phosphorate analogs, alkyl- and aryl- phosphonothioates, phosphorothioate or phosphorodithioate analogs of oligonucleotides, phosphoramidate analogs of oligonucleotides, neutral phosphate ester oligonucleotide analogs, such as phosphotriesters and other oligonucleotide analogs and modified oligonucleosides, and also includes nucleoside/non-nucleoside polymers. The term also includes nucleoside/non-nucleoside polymers wherein one or more of the phosphorus group linkages between monomeric units has been replaced by a non-phosphorous linkage such as a formacetal linkage, a thioformacetal linkage, a sulfamate linkage, or a carbamate linkage. It also includes nucleoside/non-nucleoside polymers wherein both the sugar and the phosphorous moiety have been replaced or modified such

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as morpholino base analogs, or polyamide base analogs. It also includes nucleoside/non-nucleoside polymers wherein the base, the sugar, and the phosphate backbone or a nucleoside are either replaced by a non-nucleoside moiety or wherein a non-nucleoside moiety is inserted into the nucleoside/non-nucleoside polymer. Optionally, said non-

5   nucleoside moiety may serve to link other small molecules which may interact with target sequences or alter uptake into target cells.

The term "alkyl- or aryl-phosphorate Oligomer" refers to Oligomers having at least one alkyl- or aryl-phosphorate internucleosidyl linkage. Suitable alkyl- or aryl-phosphorate groups include alkyl- or aryl- groups which do not sterically hinder the

10   phosphorate linkage or interact with each other. Preferred alkyl groups include lower alkyl groups having from about 2 to about 6 carbon atoms. Suitable aryl groups have at least one ring having a conjugated pi electron system and include carbocyclic aryl and heterocyclic aryl groups, which may be optionally substituted and preferably having up to about 10 carbon atoms.

15   The term "methylphosphonate Oligomer" (or "MP-Oligomer") refers to Oligomers having at least one methylphosphonate internucleosidyl linkage.

The term "neutral Oligomer" refers to Oligomers which have nonionic internucleosidyl linkages between nucleoside monomers (i.e., linkages having no positive or negative ionic charge) and include, for example, Oligomers having internucleosidyl

20   linkages such as alkyl- or aryl- phosphorate linkages, alkyl- or aryl-phosphonothioates, neutral phosphate ester linkages such as phosphotriester linkages, especially neutral ethyltriester linkages; and non-phosphorus-containing internucleosidyl linkages, such as sulfamate, morpholino, formacetal, thioformacetal, and carbamate linkages. Optionally, a neutral Oligomer may comprise a conjugate between an oligonucleotide or

25   nucleoside/non-nucleoside polymer and a second molecule which comprises a conjugation partner. Such conjugation partners may comprise intercalators, alkylating agents, binding substances for cell surface receptors, lipophilic agents, nucleic acid modifying groups including photo-cross-linking agents such as psoralen and groups capable or cleaving a targeted portion of a nucleic acid, and the like. Such conjugation

30   partners may further enhance the uptake of the Oligomer, modify the interaction of the

Oligomer with the target sequence, or alter the pharmacokinetics distribution of the Oligomer. The essential requirement is that the Oligonucleotide or nucleoside/non-nucleoside polymer that the Oligomer conjugate comprises be substantially neutral and capable of hybridizing to its complementary target sequence.

5   The term "substantially neutral" in referring to an Oligomer refers to those Oligomers in which at least about 80 percent of the internucleosidyl linkages between the nucleoside monomers are nonionic linkages.

The term "neutral alkyl- or aryl- phosphorate Oligomer" refers to neutral Oligomers having neutral internucleosidyl linkages which comprise at least one alkyl-
10   or aryl- phosphorate linkage.

The term "neutral methylphosphonate Oligomer" refers to neutral Oligomers having internucleosidyl linkages which comprise at least one methylphosphonate linkage.

The term "tandem oligonucleotide" or "tandem Oligomer" refers to an oligonucleotide or Oligomer which is complementary to a sequence located either on the
15   5'- or 3'- side of a target nucleic acid sequence and which is co-hybridized with a second Oligomer which is complementary to the target sequence. Tandem Oligomers may improve hybridization of these Oligomers to the target by helping to make the target sequence more accessible to such Oligomers, such as by decreasing the secondary structure of the target nucleic acid sequence. In addition, one member of a pair of
20   tandem Oligomers may improve the hybrid stability of the second tandem Oligomer to the target nucleic acid sequence by promoting a helical structure at either the 5'- or 3'- end of said second Oligomer and vice-versa.

A preferred therapy is a combination of antisense molecules targeted against a cyclin and a cyclin dependent kinase. By "antisense molecules against", "antisense
25   sequences against", "antisense against", and similar terms is meant an oligomer complementary to a nucleic acid target sequence for a particular cyclin or cyclin dependent kinase which oligomer will prevent or interfere with expression or function of the particular cyclin or cyclin dependent kinase. Certain combinations of cyclins and cyclin dependent kineses that are reported to be involved in smooth muscle proliferation
30   and restenosis. In the transition of cells from G1 to S-phase, the early stages of smooth

muscle proliferation in response to injury in the vessel wall, the kinase cdk2 is reported to be activated by a combination of cyclins A and E which appear late in G1 (Koff, et al., Science, 1992 257:1689). Accordingly, a combination preferred of antisense molecules to decrease or inhibit restenosis would be either a combination of antisense

5   oligomers against cyclin A and cdk2 or cyclin E and cdk2. Cyclin P46 or cyclin X (Williams et al. J. Biol. Chem. ,1993 268:8871) has been reported to appear in mid G1 and to activate cdk2 ahead of cyclins A and E. Thus, another preferred combination would be the combination of antisense against cyclin X and cdk2. The cell cycle dependent kinase cdk4 has been reported to be activated earlier in G1 than in cdk2, and

10   appears to be important in the G1-S-phase transition. Cdk4 has been reported to be activated by the inductions of cyclins D2 and D3. (Matsushime, et al. Cell, 1992. 71:323). We believe the combinations of antisense sequences against D2 and cdk4, or D3 and cdk4 are also preferred.

         Once cells pass through the G1-S-phase transition and replicate their DNA, they

15   are termed "competent". Cell division can be inhibited by preventing synthesis or conversely by inhibiting the later stage transition from G2 to M-phase termed "mitosis". Cdc2 kinase controls "progression" and it is activated by complexing with the cyclins B1, B2, and A. (Parker and Pinwoica-Worms, Science, 1992. 257:1955). Thus, a preferred combinations of antisense oligomers to inhibit mitosis would be a cyclin B -

20   cdc2, or a cyclin A-cdc2. Since the induction of cyclins has been reported as a key event in activation of cyclin-kinase complex leading to transition of cells into and through the cell cycle (Ohtsubo and Roberts, Science, 1993, 259:1908). Cyclins D-type, D2 and D3 which have been reported to activate cdk4 (Ajchenbaum et al., J. Biol. Chem. ,1993, 268:4113) are reported to be induced by growth factors. Accordingly,

25   antisense oligomers to these proteins may be employed according to the methods of the present invention.

         The net effect of induction of cyclins is the activation of the nuclear kinase complexes leading to phosphorylation of nuclear proteins and then liberation of free transcription factors such as E2F that promote expression of genes that are important in

30   replication of DNA and mitosis. O'Connor et al. (J. Biol. Chem., 1993. 268:8298)

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have reported that the nitrogen mustard drugs, known inhibitors of cell divisi n, work by specifically interfering with the cyclin A-cdk2 kinase complex and the cyclin B1-cdc2 kinase complex. It is believed that combinations of antisense molecules which interfere with the cyclin A-cdk2 complex will prevent DNA synthesis and molecules that interfere

5   with the cyclin B1-cdc2 complex will interfere with mitosis.

A preferred combination is an antisense sequence against proliferating cell nuclear antigen (PCNA) and an antisense sequence to cyclin or a cyclin dependent kinase and most particularly an antisense sequence against PCNA and cdc2. Antisense sequences complimentary to the mRNA of the proteins that express basic fibroblast

10   growth factor (bFGF), transforming growth factor-beta (TGF-β), and platelet derived growth factor (PDGF) alone or in combination with the above cyclins or cyclin dependent kinases are useful in practicing this invention.

Methods of Application of the Oligonucleotides

15   The antisense sequences may be administered in any convenient vehicle, which is physiologically acceptable. For the most part liquid media will be employed, such as saline, phosphate buffered saline, aqueous ethanol, or other media composition compatible with the oligomers and the tissue or cells to be treated. In some instances liposomes may be employed to facilitate uptake of the antisense oligonucleotide into the

20   target vascular smooth muscle cells, particularly where the liposome is bound to ligands for target cell receptors, which provide for endocytosis of the subject compositions. Hydrogels may also be used as depots for placement at the site of the lesion. See, for example, WO 93/01286.

Of particular interest is the use of cationic liposomes for use in cationic

25   liposome-mediated transfection (lipofection) (see Itoh, et al. Hypertension 16, 325 (abstract) (1990)) or neutral liposomes. Concentrations of liposome will generally range from about 1-5, preferably 2-4 μg/ml in the medium, where the ratio of liposome to DNA will generally be about 1:3-1:10 (w/w), preferably about 1:6 (w/w). Neutral liposomes also find application, where mono or diester glycerides (fatty acids of from

30   8-24 carbon atoms, saturated or unsaturated), particularly phosphatidyl serine,

phosphatidyl choline, etc., cholesterol, or other neutral liposome-forming monomers may find use. Moreover, complexing or covalent binding to the liposomes of proteins which aid in the binding and uptake of the liposomes find use, such as viral coat proteins, monoclonal antibodies to surface membrane proteins, and the like. The

5   manner of binding and the particular proteins will vary depending on the target tissue, manner of administration, protein availability, stability, level of immunogenicity and the like.

The invention is useful in preventing proliferation of smooth muscle cells in a variety of vascular procedures such as coronary artery angioplasty, renal artery

10   angioplasty, carotid artery surgery, renal dialysis fistulae stenosis, and vascular graft stenosis.

The antisense sequence is in a pharmaceutically acceptable compositions and is applied directly to the vascular site of injury.

Kato, et al., The Journal of Biological Chemistry, Vol. 266, No. 6, Feb. 25, pp.

15   3361-3364 (1991), Kaneda et al., Science, Vol. 243 pp. 375-378 (1988) describe liposomes containing the hemagglutinating virus of Japan (HVJ) as a vehicle for delivering DNA to cells. The HVJ containing liposomes have been found to be particularly useful vehicles for localized delivery of the antisense sequences of this invention.

20   Whenever possible, administration of the subject compositions will be localized, either by virtue of cell surface recognition markers, or by the manner of administration, such as catheter, syringe, placement of a depot, and the like. In some instances, the subject compositions may be infused upstream from the site of the cells whose activity is to be modulated. The agent can be introduced to the vessel wall luminally,

25   intramurally or periadventitially to localize the agent at the site of the lesion. One preferred method is to deliver the antisense drug through a catheter placed in the coronary artery at the site of the lesion following the angioplasty. The localized concentration or amount administered may be determined empirically and will depend upon the purpose of the administration, the area to be treated, the effectiveness of the

composition, the manner of administration, and the like. The local concentration will desirably be in the range of about 0.1 to 50 μM.

The following examples are offered by way of illustration and not by way of limitation of the present invention.

EXPERIMENTAL

Example 1

Growth of Vascular Smooth Muscle Cells in Culture

Rat aortic smooth muscle cells (passage 5-10) isolated and cultured according to
the method of Owens, et al. (J. Cell. Biol., 102, 343-352 [1986]), were plated into 24-
well culture dishes at 1 x 10⁴ cells/well. At confluence, the cells were made quiescent
by incubation for 48 hours in a defined serum-free (DSF) medium containing insulin (5
x 10⁻⁷ M), transferrin (5 μg/ml), and ascorbate (0.2 mM). This growth condition
maintains smooth muscle cells in a quiescent, non-catabolic state and promotes the
expression of smooth muscle cell-specific contractile proteins.

Synthesis and Purification of Oligomers

Oligonucleotide sequences utilized in this study and their relationships to TGF-β_1,
bFGF, and PDGE A chain mRNAs (Sporn, et al., J. Cell Biol. 105, 1039-1045 [1987];
Burgess and Maciag, Ann. Rev. Biochem. 58, 575-606 [1989]; Betsholz, et al., Nature
320, 695-699 [1986] are shown below.

Unmodified, 15-base deoxyribonucleotides were synthesized on an automated
solid-phase synthesizer (Applied Biosystems Incorporated) using standard phosphoramide
chemistry. Prior to use, the oligomers were purified by gel filtration, ethanol-
precipitated, lyophilized to dryness and dissolved in the culture media. Antisense TGF,
antisense FGF and antisense PDGF oligonucleotides were complementary to human
TGF-β_1 mRNA, bFGF and mRNA, and PDGR A chain mRNA, respectively, at the
translation initiation region. Control oligonucleotides were either the sense
oligonucleotide (sense TGF, sense FGF, sense PDGF) or the oligonucleotide with the
same oligonucleotide sequence but with a reversed 5'-3' orientation (reverse TGF,
reverse FGF). To introduce the oligonucleotides into VSMC, a cationic liposome-
mediated transfection method (lipofection) was employed (Itoh, et al. Hypertension 16,
325 [abstract] [1990]; Nabel, et al., Science 249, 1285-1288 [1990]). Oligonucleotides
dissolved in 50 μl DSF media were mixed with Lipofectin™ Reagent DOTMA (N[1-(2,3-
dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) (BRL Life Technologies,

Gaithersburg, MD) dissolved in the same volume of water in a rati of 6/1 (w/w) and
incubated for 30 minutes at room temperature. The oligonucleotides/lipsome complex
(100 μl) was then added dropwise t each well.

<u>Bioassay for TGF-β</u>

CCL-64 mink lung epithelial cells (Danielpour, et al., <u>J.Cell. Physiol</u>. 138, 79-86 [1989] were maintained in MEM supplemented with 10% fetal calf serum and 0.1 mM nonessential amino acids. Cells were spread at a density of 4 x 10^4 cells/well in 24-well plates one day before the assay. The subconfluent cells were washed once and fed with DSF containing vehicle or TGF-β (human TGF-β_1, R & D Systems, Minneapolis, MN). Twenty hours later, the cells were pulsed for 8 hours with ^{3}H-thymidine (10 μ Ci/ml). The incorporation of ^{3}H-thymidine was determined as described below and expressed as the percent of incorporation of the control (without TGF-β) wells. The levels of TGF-β, secreted from quiescent VSMC in a 14-hour period were similarly assayed at four dilutions.

Demonstration that the inhibitory effects of the conditioned media were due to TGF-β was accomplished by blocking the growth inhibitory effect with a neutralizing antibody. Fresh DSF media, human TGF-β_1 (2 ng/ml) in DSF media, or the conditioned media collected from VSMC were incubated at 37°C for 1 hour with either turkey preimmune serum or turkey anti-human TGF-β_1 antiserum (Danielpour, et al., 1989, *supra*) (1/200 final dilution prior to the addition to the CCL-64 mink lung epithelial cell bioassay at a 1/2 dilution. This treatment completely abolished the growth inhibitory action of the conditioned media (Gibbons, et al., <u>Clin. Res.</u> 38, 287a [1990]).

<u>Bioassay for bFGF</u>

Extraction of bFGF from VSMC and bioassay for bFGF activity using mouse 3T3 fibroblasts were performed as in a previous report (Klagsbrun, et al., <u>Proc. Natl. Acad. Sci. USA</u> 83. 2448-2452 [1986]). Confluent quiescent rat VSMC (1.3 x 10^{-7}) with or without previous treatment with antisense FGF-oligonucleotides were harvested from monolayer cultures by trypsinization, washed with phosphate-buffered saline, (1 μg/ml), pepstatin (4 μM) and phenylmethylsulfonyl fluoride (1 nM). After cells were disrupted by 3 cycles of freezing and thawing followed by sonication for 1 minute, the homogenate was centrifuged at 25,000xg for 30 minutes and the supernatant was

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dialyzed overnight against 0.1 M NaCl/0.01 M Tris-HCl, pH 7.5. All procedures were performed at 4°C, and aliquots f cell extracts were stored at -80°C until use. For the measurement of bFGF activity, human bFGF standards 0.03-3 ng/ml, Genzyme Corporation, Boston, MA) or samples pre-incubated (2 hours at 37°C) with either anti-

5   bFGF IgG (R & D Systems, Minneapolis, MN) or non-immune IgG at 10 μg/ml were incubated with quiescent Swiss 3T3 cells for 20 hours, after which the cells were pulse-labeled with 10 μCi/ml ³H-thymidine for 8 hours. Addition of non-immune IgG had no effect on basal or bFGF-stimulated thymidine incorporation, nor did the administration of anti-bFGF IgG affect basal thymidine uptake. Anti-bFGF IgG (10 μg/ml) almost

10   completely abolished the mitogenic activity of 1 ng/ml recombinant human bFGF, without affecting the mitogenic activity of acidic FGF or PDGF. Serial dilution curves of cell extracts were parallel to the standard curve of bFGF, and cellular bFGF content was estimated by antibody-suppressible mitogenic activity in the samples.

15   Determination of DNA Synthesis

     Relative rates of DNA synthesis were assessed by determination of tritiated thymidine (10 μCi/ml) incorporated into trichloroacetic acid (TCA)-precipitable material as previously reported (Itoh, et al., J. Clin. Invest. 86, 1690-1697 [1990]).

20   Results

     Ang II was shown to induce a 2.5-fold increase in the VSMC synthesis and release of biologically-active TGF-β using the mink lung epithelial cell bioassay. In this assay, active TGF-β caused a dose-dependent inhibition of DNA synthesis of the cells (IC_{50} = 8 x 10^{-11} M). The growth inhibitory activity in the conditioned media of the

25   VSMC could be abolished by prior incubation of the media with a specific TGF-β neutralizing antibody. This demonstrated the specificity of the assay for TGF-β.

     The synthesis by VSMC of bFGF was shown to be stimulated by Ang II. Messenger RNA for bFGF was induced modestly by Ang II (approximately 2-fold), while antibody-inhibitable bFGF activity in cell extracts, as detected by the Swiss 3T3

30   cell bioassay was increased 3-fold. In this assay, confluent, quiescent VSMC are

exposed to vehicle (basal) or Ang II (10^{-6} M) and the cells extracted and assayed for antibody-inhibitable bFGF activity using Swiss 3T3 fibroblasts as bioassay. The standard curve demonstrated the dose-dependent simulation of DNA synthesis by bFGF. Extracts from Ang II-treated cells contained 2-3-fold higher bFGF activity.

5   Antisense oligonucleotides (15 mer) complementary to human TGF-β_1 bFGF and PDGF A chain mRNAs as well as control oligonucleotides were synthesized. See Fig. 1.

The oligonucleotides were introduced into VSMC by cationic liposome-mediated transfection (lipofection). The optimum *in vivo* concentration of cationic liposome and 10 its ratio to DNA that minimized cell toxicity and optimized DNA uptake were determined to be 2-4 μg/ml and 1:6 (w/w), respectively.

To determine the effect of antisense oligonucleotides on the production of TGF-β and bFGF, confluent quiescent VSMC were treated with antisense or control oligonucleotides (5 μM) directed against TGF-β or bFGF. 50 and 250 μl of conditioned 15 media were collected after 20 h and assayed for TGF-β activity using the mink lung epithelial cell bioassay. Antisense oligonucleotides resulted in a 75% decrease in TGF-β activity. bFGF content in extracts of control or antisense oligomer-treated VSMC was assayed using the Swiss 3T3 bioassay. The antisense oligomer inhibited bFGF production by 85%.

20   Extracts from quiescent VSMC contained 2.9 ng of bFGF per mg protein. This quantity was unaffected by incubation with control oligonucleotide but was decreased to below detectable levels (< 1 ng/mg protein) when the cells were incubated with 4 μM antisense oligomer for 24 h.

In a companion study, the growth of cultured endothelial cells, which utilized 25 bFGF as an autocrine growth factor, was also inhibited effectively with antisense oligomers directed against bFGF.

In the next study, the effects of the antisense TGF-β oligonucleotide on DNA synthesis in basal and Ang II-stimulated VSMC were determined. The effect of antisense oligomers on VSMC proliferation was determined by transfecting confluent 30 quiescent VSMC with antisense or control oligonucleotides (5 μM) 4 hours prior to

addition of vehicle r Ang II (10^{-6} M). After 20 hours, the cells were labeled with 10 μCi/ml ^{3}H-thymidine f r 8 hours. Thymidine incorporati n was then determined by measuring the radioactivity of the cells. TGF-β antisense oligomer (5 μM) potentiated DNA synthesis by 35% in basal state, but more significantly in Ang II-stimulated state (87%, $p > 0.05$). In contrast, there was no change in the rate of DNA synthesis in Ang-II-stimulated cell groups transfected with the control oligomer. The results indicate that TGF-β exerts a tonic inhibitory action on VSMC proliferation and that in the Ang II-stimulated state, it plays an even greater role in growth inhibition. The antisense TGF treatment also causes a significant reduction of RNA synthesis (^{3}H-uridine incorporation) by 60% at the basal state. These results support the conclusion that endogenous production of TGF-β exerts a hypertropic and antiproliferative action.

The effect of the antisense oligomer to bFGF employed at 5 μM was shown to suppress DNA synthesis in the basal state and inhibited this process significantly by (30%) in the Ang II-stimulated state. bFGF acts as a promoter of VSMC proliferation, especially in the Ang II-stimulated state. In contrast, antisense oligomers directed against PDGF-A had no effect on basal or Ang II-stimulated ^{3}H-thymidine incorporation. The data supports the conclusion that Ang II activates a growth-stimulatory pathway mediated primarily by bFGF.

Co-transfection of bFGF antisense oligomer with TGF-β antisense oligomer almost completely abolished Ang II-induced VSMC proliferation. The co-transfection of bFGF antisense oligomer with TGF-β antisense oligomer almost completely abolished Ang II VSMC proliferation that was unmasked by the blockade of TGF-β production. In contrast, transfection of the antisense directed against PDGF-A had no effect on the DNA synthesis uncovered by the antisense oligomer directed against TGF-β.

The above data supports the conclusion that the bFGF and TGF-β growth factor counteract within the same population in an autocrine and/or intracrine fashion. Since bFGF is known to promote the production of plasminogen activator, which is crucial for the activation of TGF-β, the interaction of these dual autocrine loops in the production and activation of these growth factors is an essential aspect of the regulation of VSMC proliferation.

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Example 2

Confluent, quiescent cells, cultured as described in Example 1, were exposed to a combination of antisense oligomers directed against cdc2 kinase, cdk2 kinase, and cyclin B (Tables 2 and 3) (4 μM each). The oligomers were synthesized as described
5 above with the exception that phosphorothiate modified nucleotides were employed. The oligomers (antisense and control) were introduced into the cells using virally-mediated liposome uptake (Kaneda, et al., J. Biol. Chem., (1989) 264, 12126-12129). To assess the effect of these oligomers on the growth of VSMC, cells were grown for 4 days after oligomer exposure. The cells were then harvested by trypsin and assayed for DNA
10 content. The results demonstrated that sense oligomer treated cultures had DNA contents indistinguishable from control untreated cultures, demonstrating that the sense oligomers had no effect on the basal growth of the VSMC cultures. On the other hand, cells exposed to the antisense oligomers exhibited a 50% decrease in DNA content, demonstrating that these oligomers decreased by 50% the basal growth of these cultures.
15   These oligomers also inhibited growth factor stimulated growth. Treatment of these cells with FGF increased the content of DNA by 4-fold. In cultures treated with FGF plus sense oligomers, DNA content increased 3-fold, demonstrating that the presence of sense oligomers had little effect on growth factor-induced increase in DNA content. However, when FGF-treated cells were exposed to the antisense oligomers,
20 no increase in the basal levels of DNA were observed, demonstrating that these oligomers blocked the growth factor induced growth of these cells.

Similar results were seen when a combination of antisense oligomers directed against cdc2 kinase and PCNA were used.

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Example 3

Growth of Vascular Smooth Muscle *In Vivo* in Response to Injury

Male Sprague-Dawley rats weighing 500-550 grams were used. Endothelial denudation and injury to the vascular wall were performed on carotid arteries. Rats

5   were anesthetized with the administration of kentamine hydrochloride (80 mg/kg) (Parke-Davis, Morris Plains, NJ) and xylazine (12 mg/kg) (Lloyd Laboratories, Shenandoah, IA). A 2 French embolectomy balloon catheter (American Edward Laboratories, Santa Ana, CA) was passed into the left common carotid via the left external carotid artery according to the previously described method of Clowes, et al.

10 (Lab Invest. 49, 208-215 [1983]). Using Evans Blue staining, we documented that this procedure denuded the endothelium effectively. The right, uninjured carotid was used as a control.

At the time of injury, some animals received a combination of antisense oligomers directed against cdc2 kinase and PCNA (tables 2 and 3). The synthesis of the

15 oligomers was as described above except that phosphothionate modified sequences were employed. The oligomers were introduced lumenally to the vessel wall. Uptake of the DNA was mediated via virally coated liposomes (Kaneda, et al., J. Biol. Chem. 264, 12126-12129 [1989]).

To determine the effect of the oligomer treatment on DNA synthesis in the

20 injured vessel, the animals were injected with BrdUr. This thymidine analogue is incorporated into the newly synthesized DNA and is detected immunohistochemically.

All rats were euthanized 4 days following injury. Rats were anesthetized and blood was withdrawn from the ascending aorta via the left verticle. The rats were then perfused at a pressure of 110 mm/Hg through the ascending aorta with 100 ml of

25 phosphate buffered saline (PBS, pH 7.2). The carotid arteries were removed and small segments were fixed in 10% formaldehyde and embedded in paraffin. Five micron sections were cut on a rotary microtome and placed on glass slides that had been treated for 20 seconds with acetone containing 2% 3-aminopropyltriethoxysilane (Sigma Chemical, St. Louis, MO). The sections were digested with 0.1% trypsin (Sigma

30 Chemical, St. Louis, MO) in PBS at 37°C for 12 minutes. The sections were then

preincubated with normal serum from the species that the second step antibody was
produced. The sections were then covered with a primary antibody specific for BrdUr,
incubated for 15 minutes at 4°C, washed with PBS and then incubated with the
biotinylated secondary antibody. After washing, the sections were incubated with
5   avidinbiotinylated horseradish peroxidase complex (Vectastain ABC kits, Vector
Laboratory, Burlingame, CA), and developed with 0.1% diaminobenzidine
tetrahydrochloride (Aldrich Chemical, Milwaukee, WI) and 0.02% $H_2 O_2$ (Sigma
Chemical, St. Louis, MO), the slides were then counter stained with hematoxylin.
Positive staining appeared as brown in color. Negative controls included the following:
10   1) omission of primary or secondary antibody, 2) replacement of the primary antibody
by non-immune serum or immunoglobulin of the same species.

The results demonstrated that control uninjured vessels exhibit very low levels
of DNA synthesis (<1% BrdUr labeling index). On the other hand, the injured
untreated vessel inhibited a high level of BrdUr staining (23% BrdUr labeling index)
15   demonstrating that vascular injury induces DNA synthesis within the vessel wall. in the
cases of exposure of these injured vessels to antisense oligomers, DNA synthesis was
decreased by 60%. Moreover, DNA content within the antisense treated vessel wall
decreased by 40% when compared to the sense treated vessel.

Neointima formation was assessed by morphometry of perfusion-fixed specimens
20   14 days after balloon injury in a blinded fashion. Our results demonstrated a dose-
dependent suppression of neointima formation by the combination of PCNA-cdc2
antisense oligodeoxynucleotide (ODN). A low dose (3 μM each) of antisense ODN
inhibited neointima formation by approximately 60% (n=7) compared to vessels treated
with the HVJ-liposome complex alone (n=5) or sense ODN-treated vessels (n=8). The
25   antisense ODN had no significant effect on medial area. At a dose of 15 μM each, a
single administration of the PCNA-cdc2 antisense ODN combination completely
abolished neointima formation (n=8), (greater than 95%) whereas sense control ODN
had no effect (n=8) Figure 3. Importantly, a significant inhibition of neointima
formation was observed up to 4 and 8 weeks after administration of the PCNA-cdc2
30   antisense ODN combination compared to sense control, Figure 3. Intraluminal

transfection of PCNA antisense ODN alone failed to inhibit neointima formation (neotintimal area=0.237 ±0.016 mm² versus sense control area=0.214±0.022 mm², p. 0.05). These data suggest that there is an amplified inhibitory effect of combined administration of both PCNA and cdc2 antisense ODN. The selectivity of the antisense

5   ODN effect was further confirmed by the observation that the inhibition of the neointima was limited to the area of intraluminal transfection. In contrast, the adjacent injured carotid segments outside the area of antisense transfection exhibited neointimal lesions similar to the sense ODN-treated control. These results are summarized in Figure 3.

The intraluminal administration of antisense cdk2 alone at 3 μM and 15 μM with

10 the HVJ method resulted in a 60% reduction in DNA synthesis as measured by BrdUr labeling as well as a 60% reduction in neointima lesion formation at 2 weeks after vascular injury (Figure 4, columns 4 and 6). As can be seen from Figure 4, column 5, the effect of administering the combination of cdk2 antisense ODN (3 μM) and cdc2 (3 μM) antisense ODN on neointima formation was not significantly different from cdk2

15 (3 μM) alone.

Since DNA synthesis in the cell of the injured vessel is required for full development of the neointimal lesion, inhibition of DNA synthesis in those cells should inhibit the lesion formation.

The above concentrations of antisense sequences to PCNA and cdc2 were

20 conveniently used to demonstrate the synergistic effect of these sequences on modifying DNA synthesis and cardiovascular cellular activity of vascular smooth muscle cells associated with formation of neointima. The desired range of antisense concentration can be determined empirically for difference antisense sequences and will vary depending on factors such as, but not limited to, the nature and location of the sequence

25 targeted by the antisense and the length of the antisense sequence.

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Example 4

This example summarizes a study performed in an attempt to enhance the efficiency of cellular uptake and the stability of antisense ODN. The study involves a viral protein-mediated ODN transfer technique.

5

Synthesis of oligomers and selection of sequence targets

The sequences of antisense oligonucleotides (ODN) against cell cycle regulatory genes used in this Example are shown in Table 4. Synthetic ODN were washed by 70% ethanol, dried, and dissolved in sterile Tris-EDTA buffer (10mM Tris, 1 mM EDTA.)

10 The supernatant was purified over NAP 10 column (Pharmacia),a nd quantitated by spectrophotometry as described in Itoh, J., Mukoyama, M., Pratt, R.e., Dzau, V.J. (1992) Biochem Biophysic Res Comm 188, 1205-1213.

Preparation of HVJ-liposomes

15   Phosphatidylserine, Phosphatidylcholone, and cholesterol were mixed in a weight ratio of 1:4.8:2 as described in Kaneda, Y., Iwai, K., Uchida, T. (1989) Science 243, 375-378; Tomita, N., Higaki, J., Morishita, R., Kato, K., Mikami, J., Kaneda Y., Ogihara, T. (1992) Biochem Biophysic Res Comm 186, 129-134; Morishita, R., Gibbons, G.H., Kaneda, Y., Ogihara, T., Dzau, V.J., J Cell Biochem; Itoh, J.,

20 Mukoyama, M., Pratt, R.e., Dzau, V.J. (1992) Biochem Biophysic Res Comm 188, 1205-1213. Dried lipid was hydrated in 200 μl of balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6) containing sense or antisense-ODN (600 nmol). Liposomes were prepared by shaking and sonication. Purified HVJ (Z strain) was inactivated by UV irradiation (110 erg/mm^2/sec) for 3 min just before use. The

25 liposome suspension was mixed with HVJ (10000 hemagglutinating units) in a total volume of 4 ml of BSS. The mixture was incubated at 4°C for 5 mnutes and then for 30 minutes with gentle shaking at 37°C. Free HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. The top layer of the sucrose gradient was collected for use. The final concentration of antisense ODN is equivalent

30 to 15 μM, as calculated according to Kaneda, Y., Iwai, K., Uchida, T. (1989) Science

243, 375-378; Tomita, N., Higaki, J., Morishita, R., Kato, K., Mikami, J., Kaneda Y., Ogihara, T. (1992) Biochem Biophysic Res Comm 186, 129-134; Morishita, R., Gibbons, G.H., Kaneda, Y., Ogihara, T., Dzau, V.J., J Cell Biochem.

5

In vivo transfer of ODN

A 2 French Fogarty catheter was used to induce vascular injury in male Sprague-Dawley rats (400-500g; Charles River Breeding Laboratories) as described in Rakugi, J., Jacob, J.K., Krieger, J.E., Ingelfinger, J.R., Pratt, R.E., (1993) Circulation 87, 283-290. The rats were anesthetized, and a cannula introduced into the left common carotid via the external carotid artery. After vascular injury of the common carotid, the distal injured segment was transiently isolated by temporary ligatures. The HVJ-liposome complex was infused into the segment and incubated for 10 minutes at room temperature. After a 10 minute incubation, the infusion cannula was removed. Following the transfection, blood flow to the common carotid was restored by release of the ligatures. No adverse neurological or vascular effects were observed in any animal undergoing this procedure.

At 2, 4 and 8 weeks after transfection, rats were sacrificed and vessels were perfusion-fixed with 4% paraformaldehyde. Three individual sections from the middle of transfected segments were analyzed. In addition, three sections from the middle section of the injured untransfected region was also analyzed. Animals were coded so that the operation and analysis were performed without knowledge of which treatment individual animals received.

A statistical analysis of the results is found in Table 5. All values are expressed as mean ± SEM. All experiments were repeated at least three times. Analysis of variance with subsequent Duncan's test was used to determine significant differences in multiple comparisons. $P < 0.05$ was considered significant.

Initially, the effect of a single antisense ODN at 15 μM on the inhibition of neointima formation in rat carotid artery model was examined. Administration of either antisense cdc 2 kinase (both mouse and rat sequences) or antisense cdk 2 kinase ODN resulted in the partial inhibition of neointima formation after balloon injury, whereas antisense PCNA ODN alone failed to show any inhibitory effects. To achieve the further inhibition a combination of these ODN were administered. Indeed, a single administration of 15 μM of the PCNA/cdc 2 (mouse sequence) antisense ODN combination completely abolished neointima formation (n=8), whereas the sense control

ODN (15 μM each) had no effect (n=8). The antisense ODN had no significant effect on medial area. The administration of combination of cdc 2 kinase (rat sequences) and PCNA ODN inhibited neointima formation almost completely.

Also examined was the combination of antisense cdc 2 kinae and syslin B ODN. A single administration of combination of antisense cdc 2 and cyclin B ODN inhibited neointima formation significantly. The selectivity of the antisense ODN effect was further confirmed by the observation that the inhibition of the neointima formation was limited to the area of intraluminal transfection along the injured carotid artery. (Table 4). In contrast, the adjacent injured carotid segments outside the area of antisense transfection exhibited neointimal lesions similar to the sense ODN-treated control injured carotid artery. Transfection of antisense thrombomodulin ODN also failed to show any inhibitory effects on neointima formation. These results summarize Table 5.

The single administration of 15 μM of the PCNA/cdc 2 antisense ODN combination significantly inhibited the extent of neointima formation for a period of 8 weeks after transfection (Fig. 5). Although there was evidence for a neointimal lesion at 4 weeks after antisense treatment, the lesion was significantly inhibited as compared to sense treatment (P<0.01). Furthermore, no further progression of the lesion was observed at 8 weeks after a single antisense administration.

The results of this example indicates that neointima lesion formation after balloon angioplasty can be prevented by a single intraluminal administration of antisense oligonucleotides directed against cell cycle regulatory genes. This therapeutic strategy fulfills the criteria needed for success, i.e., a proper drug target, an efficient drug delivery method and an intraluminal approach.

In defining the strategy to prevent restenosis in humans, the method of drug delivery is critically important. Intraluminal delivery is desirable since it can be carried out concomitantly with the transluminal angioplasty --a practical issue of clinical relevance. The blockade of cell cycle progression is particularly attractive since it maintains the cells in a quiescent, differentiated phenotype without inducing cell injury. The HVJ-OFN intraluminal method substantially increases the efficiency of uptake and the stability of ODN in vivo thereby avoiding the potential toxicity of high

concentrations of ODN (*i.e.* > 150 uM). The modification of antisense ODN
pharmacokinetics by use of the HVJ-liposome complex will facilitate the potential
clinical utility of these agents by: 1) allowing for an abbreviated intraluminal incubation
time to preserve organ perfusion, 2) prolonging the duration of biological action, 3)
enhancing specific activity.

Example 5

This example examines the fate of antisense oligodeoxynucleotides (ODN) in vivo using high efficient viral mediated transfer method (HVJ). Direct transfer of unmodified FITC-labeled ODN showed fluorescence in the medial layer, and disappeared within 1 day. In contrast, transfected unmodified FITC-ODN by HVJ method showed much florescence in the medical layer, and florescence continued at least 1 week (and disappeared within 2 weeks). Moreover, transfer of phosphorothioate FITC-ODN enwrapped in liposomes without HVJ particle resulted in a short existence of florescence (within 4 days), while transfer by HVJ method result in continuous florescence up to 2 weeks after transfection. Given that the HVJ method prolonged the half-lives of ODN the efficiency of antisense-phospohorothioate ODN against cdk kinase gene to balloon injury rat carotid artery was also examined. The specificity of antisense cdk 2 ODN was confirmed by the observation that nRNA of cdk 2 kinase in injury vessels was markedly diminished by the antisense ODN treatment. At 2 weeks after transfection, antisense cdk 2 ODN treatment resulted in a significant inhibition in neointima formation, while sense ODN treatment and untreatment did not result in reduction. On the other hand, administration of antisense cdc 2 ODN also partially inhibited neointima formation. Moreover, a single intraluminal administration of combination of antisense cdc 2 and cdk 2 ODN showed further neointima inhibition as compared to antisense cdc 2 ODN alone. This example demonstrates that a single intraluminal administration of antisense ODN directed cell cycle regulatory genes such as cdc 2 and cdk 2 kinase with HVJ method prevented neointima formation after balloon angioplasty in rat carotid injury model via a catheter delivered system.

Synthesis of oligomers and selection of sequence targets

The sequences of ODN against human cdk 2 kinase used in this study was following: antisense: 5'-GAA-GTT-CTC-CAT-GAA-GCG-3' [SEQ ID NO:41], sense: 5'-CGC-TTC-ATG-GAG-AAC-TTC-3' [SEQ ID NO:40] (-6 - +12 of human sequence; these sequences are no different between mouse and human cdk 2 kinase). This antisense ODN specifically inhibit cdk2 kinase protein synthesis and serum stimulated

growth in rat VSMC. Also synthesized were sense and antisense cdc 2 kinase ODN (antisense: 5'-GTC-TTC-CAT-AGT-TAC-TCA [SEQ ID NO:37], sense: 5'-TGA-GTA-ACT-ATG-GGA-GAC-3' [SEQ ID NO:36], -9 to +9 of mouse sequence), as described in Furukawa, U., Piwnica-Worms, H., Ernst, T.J. Kanakura, Y., Griffin, JJ.D. (1990)
5   Science 250, 805-808. Synthetic ODN were purified over NAP 10 column (Pharmacia), and quantitated by spectrophotometry.

Preparation of HVJ-liposomes

        Phosphatidylserine, Phosphatidylcholone, and cholesterol were mixed in a weight ratio of 1:4.8:2. Dried lipid was hydrated in 200μl of balanced salt solution (BSS; 137

5   nM NaCl, 5.4 nM KCl, 10mM Tris-HCl, pH7.6) containing sense of antisense-ODN (120 nmol). Control group was used as no ODN (BSS 200 ul). Liposomes were prepared by shaking and sonication. Purified HVJ (Z strain) was inactivated by UV irradiation (100 erg/mm^2/sec) for 3 min just before use. The liposome suspension (0.5 ml, containing 10 mg of lipids) was mixed with HVJ (10000 hemagglutinating units) in

10   a total volume of 4 ml of BSS. The mixture was incubated at 4°C for 5 min and then for 30 min with gently shaking at 37°C. Free HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. The final concentration is equivalent to 3 μM. In this study, the preparation of HVJ complex was optimized to achieve the maximum effect of antisense ODN in VSMC.

15

In vivo gene transfer

        A 2 French Fogarty catheter was used to induce vascular injury in male Sprague-Dawley rates (400-500g; Charles River Breeding Laboratories). These rats were anesthetized with ketamine, and the left common carotid artery was surgically exposed.

20   A cannula was introduced into the common carotid via the external carotid artery. In vivo gene transfer was assessed under following condition: Vascular injury of the common carotid was induced by the passage and inflation of a balloon catheter through an arteriotomy in the external carotid artery three times. The injured segment was transiently isolated by temporary ligatures. The HVJ-liposome complex, liposome

25   complex without HVJ particles, or ODN alone was infused into the segment and incubated for 10 minutes at room temperature as described above. After a 10 minute incubation, the infusion cannula was removed. Following the transfection, blood flow to the common carotid was infusion by release of the ligatures, and the wound was then closed. No adverse neurological or vascular effects were observed in any animal

30   undergoing this procedure.

In vivo transfection of FITC labeled ODN

FITC labeled unmodified and phosphorothioate antisense ODN were kindly
provided by Clontech Inc. (Palo Alto, CA.) FITC was labeled to 3' and 5' of ODN (16
mer). Animal surgery was performed as described above. Transfer of unmodified
5   ODN was performed under two protocols. (1) HVJ complex with FITC labeled
unmodified ODN (3μM) was incubated for 10 minutes. (2) ODN alone: unmodified
ODN 30 μM was incubated for 10 minutes. Transfer of phosphorothioate ODN was
also performed under two protocols; (1) HVJ complex with FITC labeled
phosphorothioate ODN (3μM) was incubated for 10 minutes. (2) ODN in liposomes
10   without HVJ particles: liposome complex with ODN (3 μM), but without HVJ particles
was incubated for 10 minutes. The vessels were harvested at 10 min, 1, 4, 7 and 14
days after transfection and fixed with 3% paraformaldehyde after perfusion of PBS.
Sections were examined by fluorescent microscopy, after stained for 5 min in erichrome
black T solution and washed twice for 3 min in PBS. Elastic fibers stained dark red
15   and are readily distinguishable from the specific FITC ODN by the treatment by
erichrome black T solution.

Reverse transcription (RT)-PCR

RNA was extracted from antisense or sense ODNs (cdk 2 kinase; 15 μM
20   wrapped in liposomes) treated injured vessels by RNAzol (Tel-Test, Inc., Texas) at 1
and 14 days after transfection. Contralateral arteries were also used in intact arteries.
Levels of cdk 2 and beta-actin mRNs were measured by reverse transcription-PCR. The
cdk   2   5'   primer   (neucleotides   -6-12   of   human   sequences)   was   5'-
CGCTTCATGGAGAACTTC-3' [SEQ ID NO:46]; the 3'primer (neucleotides 340-357)
25   was 5'-ATGGCAGAAAGCTAGGCC-3' [SEQ ID NO:47]. The primers complementary
to the rat beta-actin gene were 5'-TTGTAACCAACTGGGACGATATGG-3' [SEQ ID
NO:48]; the 3' primer was 5'-GATCTTGATCTTCATGGTGCTAGG-3'[SEQ ID
NO:49] (Clontech Laboratories Inc., Palo Alto, CA). Extreme care was taken to avoid
contamination of tissue samples with trace amounts of experimental RNA. Aliquots of
30   RNA derived from intact and injured vessels were amplified simultaneously by PCR (35

cycles) and compared with a negative control (primers without RNA). Amplification products were electrophoresed through 2% agarose gels stained with ethidium bromide.

In vivo transfection of antisense ODN

5   After balloon injury as described above, 500 μl of HVJ-liposomes complex containing sense or antisense cdk 2 kinase or cdc 2 kinase ODN (3 or 15 μM wrapped in liposome) was incubated within lumen for 10 minutes. Direct transfer (cdk 2 ODN; 150 μM) and liposome-mediated transfer without HVJ particle (cdk 2 ODN; 15 μM) were also examined to rat injured carotid arteries. At 2 weeks after transfection, rats

10   were sacrificed and vessels were perfusion-fixed with 4% paraformaldehyde. Neointima formation was assessed by morphometry, by individuals who were blinded to the identity of the samples. Intimal and medial areas were measured on a digitizing tablet (southern Micro Instruments, model 2200, Atlanta, GA).

15   *Statistical analysis*

All values are expressed as mean ± SEM. All experiments were repeated at least three times. Analysis of variance with subsequent Duncan's test was used to determine significant differences in multiple comparisons. $P < 0.05$ was considered significant.

20

RESULTS

Localization of FITC labeled unmodified and phosphorothioate ODN

Direct transfer of FITC unmodified ODN (30 μM) without HVJ method into rat carotid arteries revealed florescence in the medial layer at 10 minutes after transfection.

25   However, this florescence disappeared 1 day after transfection. In contrast, transfer of FITC unmodified ODN (3 μM) by HVJ method resulted in much wider florescence than direct transfer without HVJ method at 10 min after transfection despite of much lower concentration of ODN. Moreover, the florescence was stimulated in the nuclei and continued up to 1 week after transfection. At 2 weeks after transfection, florescence

30. could not be observed in the medial layer.

A comparison of the transfer of FITC labeled phosphorothioate ODN-liposome complex with or without HVJ particles, is shown in Fig. 8A. Florescence in ODN-liposome (3 μM) without HVJ particles transfected vessels was mainly seen in the media layer, but also in the adventitia at 10 min after transfection, similar to direct transfer.

5   This florescence was markedly diminished at 1 day after transfection and disappeared within 4 days. Transfer of FITC labeled phosphorothioate ODN (3 μM) by HVJ method resulted in much wider florescence which was seen at the medial and adventitial layer, and continued up to 2 weeks. This prolongation of florescence was due to the modification of backbone of ODN to phosphorothioate because of their resistance to

10   nuclease. These findings support the utility of phosphorothioate ODN in antisense strategy. At 1 and 2 weeks after transfection, florescence was mainly found in the medial layer, but not in the neointima lesions. The florescence could be observed in the nuclei at this stage. Untreated of HVJ complex without ODN treated vessels revealed no specific florescence, except autoflorescence in elastic lamina. Florescence resulted

15   by FITC labeled ODN was easily distinguished from autoflorescence. Incubation of free FITC in the vessels did not also result in specific florescence suggesting that this florescence was specific to FITC labeled ODN. Table 6 summarize these results.

Table 6. Summary of FITC labeled ODN transfection

20

time-course	direct	HVJ-unmodified	liposome	HVJ-S
10 min	+	+	+	+
1 days	nd	+	±	+
4 days	nd	+	nd	+
7 days		+		+
14 days		nd		+

nd = not detected, ± = few florescence, + = florescence

direct = direct transfer (30 μM), HVJ-unmodified = unmodified ODN by HVJ method (3 μM), liposome = liposome mediated transfer of phosphoroth iate ODN (3 μM) without HVJ particle, HVJ-S = phosphorothioate ODN (3 μM) by HVJ method.

5   *Reverse-transcriptase PCR of cdk 2 kinase mRNA expression in vivo*

        mRNA expression of cdk 2 kinase in rat carotid injury model was examined to evaluate the specificity of antisense cdk 2 ODN.   RNA from either the vessels transfected antisense or sense cdk 2 ODN (15 μM) was amplified by RT-PCR. At 1 day after injury, RT-PCR of RNA from injured vessels transfected with sense ODN

10   demonstrated a single band corresponding to cdk 2 kinase nRNA, whereas from intact vessels did not. However, administration of cdk 2 ODN abolished the increased mRNA. At least 14 days after injury, RT-PCR of RNA from vessels transfected neither sense nor antisense ODN demonstrated the detectable level of cdk 2 mRNA.   In contrast, mRNA of beta-actin was readily detected in the same protocol.

15

      Effect on antisense cdk 2 and cdc 2 kinase ODN

        Antisense cdk 2 kinase phosphorothioate ODN was applied to rat injured vessels given the phosphorothioate ODN could be stabled within 2 weeks in medial layer by HVJ transfer method, .   Untreated and sense ODN (2 and 15 μM) transfected vessels

20   exhibited neointimal formation at 2 weeks after transfection.   In contrast, a single administration of antisense cdk 2 kinase ODN (3 μM) resulted in a significant reduction in neointima formation (approximately 60% as to compared to sense ODN treated vessels). At high dose of 15 μM, antisense cdk 2 ODN resulted in a further inhibition of neointima formation, but not significant. Those treatment did not alter the medial

25   areas, suggesting that media viability was not affected by HVJ transfer method. This reaction in neointima formation was limited in transfected lesions, but not in untransfected lesions in the same animals (neointimal/medial areas ratio; transfected region = 0.597 $\pm$ 0.131 VS untransfected region - 1.156 $\pm$ 0.079, P<0.01). To investigate the contribution of HVJ transfer to the enhanced lives of ODN in vivo, the

30   biological action of antisense cdk 2 ODN among the direct transfer, liposome mediated

transfer without HVJ particle, was compared to the HVJ method. Neither direct transfer (150 μM) nor liposome mediated transfer without HVJ particle (15 μM) failed to show any inhibitory effect of antisense ODN.

5   (1) antisense cdc 2 kinase ODN alone (15 μM), and (2) combination of cdc 2 and cdk 2 kinase ODN (15 μM each) were administered investigate the antisense strategy blocking cell cycle regulatory genes. Administration of antisense cdc 2 kinase ODN partially inhibited neointima formation as well as cdk 2 ODN alone, whereas sense cdc ODN did not. The combination of cdc 2 and cdk 2 kinase ODN showed enhanced inhibition of neointima formation as compared to antisense cdc 2 or cdk 2 kinase ODN

10   alone.

This example demonstrates that HVJ method prolonged the existence of florescence in the medial layer up to 1 week (unmodified ODN) and 2 weeks (phosphorothioate ODN) after transfection of FITC-labeled ODN, respectively. These findings indicate that the HVJ method bypasses endocytosis. This is probably due to the

15   anticipation of HVJ particles, but not to liposomes, as ODN-liposome complex without HVJ particles did not result in such a prolongation. Moreover, the stimulation of florescence in the nuclei that could be seen only by HVJ method may increase the specificity of antisense ODN.

SEQUENCE LISTING

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  (iii) NUMBER OF SEQUENCES: 49

   (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

    (v) CURRENT APPLICATION DATA:
        APPLICATION NUMBER: PCT/US94/

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: mRNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCCUCCCCCA UGCCGCCCUC CGGG   24

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGAGGGCGGC ATGGG   15

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCCATGCCGC CCTCC   15

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGTACGGCG GGAGG   15

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 27 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: mRNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCAGCAGGGA CCAUGGCAGC CGGGAGC   27

(2)   INFORMATION FOR SEQ ID NO:6:

      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 15 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGCTGCCATG GTCCC   15

(2)   INFORMATION FOR SEQ ID NO:7:

      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 15 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGACCATGG CAGCC   15

(2)   INFORMATION FOR SEQ ID NO:8:

      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 15 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCCTGGTACC GTCGG   15

(2)   INFORMATION FOR SEQ ID NO:9:

      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 24 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear

        (ii) MOLECULE TYPE: mRNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

    CGGGACGCGA UGAGGACCUU GGCU   24

    (2) INFORMATION FOR SEQ ID NO:10:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 15 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

    AGCCAAGGTC CTCAT   15

    (2) INFORMATION FOR SEQ ID NO:11:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 15 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

    ATGAGGACCT TGGCT   15

    (2) INFORMATION FOR SEQ ID NO:12:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 24 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: mRNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

    UGAGUAACUA UGGAAGACUA UAUC   24

(2)   INFORMATION FOR SEQ ID NO:13:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 18 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: cDNA

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTCTTCCATA GTTACTCA   18

(2)   INFORMATION FOR SEQ ID NO:14:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 18 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: cDNA

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGAGTAACTA TGGAAGAC   18

(2)   INFORMATION FOR SEQ ID NO:15:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 30 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: mRNA

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:15:

AACUCCGCCA CCAUGUUUGA GGCAGGCCUG   30

(2)   INFORMATION FOR SEQ ID NO:16:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 15 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTCAAACATG GTGGC   15

(2) INFORMATION FOR SEQ ID NO:17:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 18 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GATCAGGCGT GCCTCAAA   18

(2) INFORMATION FOR SEQ ID NO:18:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 15 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GCCACCATGT TTGAG   15

(2) INFORMATION FOR SEQ ID NO:19:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 18 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TTTGAGGCAC GCCTGATC   18

(2)   INFORMATION FOR SEQ ID NO:20:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 21 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: mRNA

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGAGCCAUGG CGCUCAGGGU C   21

(2)   INFORMATION FOR SEQ ID NO:21:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 18 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: cDNA

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:21:

CCTGAGCGCC ATGGCTCC   18

(2)   INFORMATION FOR SEQ ID NO:22:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 18 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: cDNA

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:22:

GGAGCCATGG CGCTCAGG   18

(2)   INFORMATION FOR SEQ ID NO:23:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 24 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

     (ii) MOLECULE TYPE: mRNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

UGGCGCUUCA UGGAGAACUU CCAA   24

(2) INFORMATION FOR SEQ ID NO:24:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 18 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GAAGTTCTCC ATGAAGCG   18

(2) INFORMATION FOR SEQ ID NO:25:
     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 18 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CGCTTCATGG AGAACTTC   18

(2) INFORMATION FOR SEQ ID NO:26:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 73 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: DNA (genomic)

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTGACTAACT ATGGAAGATT ATACCAAAAT AGAGAAAATT GGAGAAGGTA CCTATGGAGT   60

TGTGTATAAG GGT   73

(2)   INFORMATION FOR SEQ ID NO:27:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 41 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: cDNA

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:27:

GGGAGCAGTG ATGTTGGGGC AACTCTGCGC CGGGGCCTGC G   41

(2)   INFORMATION FOR SEQ ID NO:28:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 73 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: cDNA

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:28:

AGAGGAAGCC ATGGCGCTCC GAGTCACCAG GAACTCGAAA ATTAATGCTG AAAATAAGGC   60

GAAGATCAAC ATG   73

(2)   INFORMATION FOR SEQ ID NO:29:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 81 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: cDNA

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:29:

GGCTGGGTCT ATGGTCGCTC CGCGGCCGTC CGCCGCGTGG TGCTTTTTTA TCAGGGAAAG   60

CTGTGTTCCA TGGCAGGGAA C   81

(2)   INFORMATION FOR SEQ ID NO:30:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 83 base pairs

            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

AGCCCCAGCC ATGGAACACC AGCTCCTGTG CTGCGAAGTG GAAACCATCC GCCGCGCGCT   60

ACCCCGATGC CAACCTCCTC AAC   83

    (2) INFORMATION FOR SEQ ID NO:31:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 78 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CTGCCCGAGT ATGGAGCTGC TGTGTTGCGA AGGCACCCGG CACGCGCCCC GGGCCGGGCC   60

GGACCCGCGG CTGCTGGG   78

    (2) INFORMATION FOR SEQ ID NO:32:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 81 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GCGGGACACC ATGAAGGAGG ACGGCGGCGC GGAGTTCTCG GCTCGCTCCA GGAAGAGGAA   60

GGCAAACGTG ACCGTTTTTT G   81

    (2) INFORMATION FOR SEQ ID NO:33:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 76 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CTCCGCCACC ATGTTCGAGG CGCGCCTGGT CCAGGGCTCC ATCCTCAAGA AGGTGTTGGA   60

GGCACTCAAG GACCTC   76

(2) INFORMATION FOR SEQ ID NO:34:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TGAGTAACTA TGGAAGAC   18

(2) INFORMATION FOR SEQ ID NO:35:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GTCTTCCATA GTTACTCA   18

(2) INFORMATION FOR SEQ ID NO:36:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

TGAGTAACTA TGGAGGAC   18

(2)   INFORMATION FOR SEQ ID NO:37:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 18 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii) MOLECULE TYPE: cDNA

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GTCTTCCATA GTTACTCA   18

(2)   INFORMATION FOR SEQ ID NO:38:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 18 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii) MOLECULE TYPE: cDNA

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TTTGAGGCAC GCCTGATC   18

(2)   INFORMATION FOR SEQ ID NO:39:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 18 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii) MOLECULE TYPE: cDNA

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GATCAGGCGT GCCTCAAA   18

(2)   INFORMATION FOR SEQ ID NO:40:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 18 base pairs
              (B)   TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CGCTTCATGG CGAACTTC   18

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 18 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GAAGTTCTCC ATGAAGCG   18

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 18 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GAAGGAGCCA TGGCGCTC   18

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 18 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GAGCGCCATG GCTCCTCC   18

(2)   INFORMATION FOR SEQ ID NO:44:

      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 19 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CTTCGTCGGT ACCGTCTTC   19

(2)   INFORMATION FOR SEQ ID NO:45:

      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 18 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

ACCCAGAAAG AAAATCCC   18

(2)   INFORMATION FOR SEQ ID NO:46:

      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 18 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CGCTTCATGG AGAACTTC   18

(2)   INFORMATION FOR SEQ ID NO:47:

      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 18 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

ATGGCAGAAA GCTAGGCC   18

(2) INFORMATION FOR SEQ ID NO:48:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

TTGTAACCAA CTGGGACGAT ATGG   24

(2) INFORMATION FOR SEQ ID NO:49:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GATCTTGATC TTCATGGTGC TAGG   24

WHAT IS CLAIMED IS:

1.   A method for inhibiting vascular cellular activity of cells associated with vascular lesion formation in mammals, said method comprising:

administering to the site associated with vascular lesion formation an effective dosage of at least one antisense oligomer sequence to at least one gene expressing a cyclin or a cyclin dependent kinase wherein the antisense sequence inhibits the expression of the gene.

2.   A method according to claim 1 wherein the cyclin is cyclin A, B1, B2, C, D1, D2, D3, E or cyclin X(p46) and the cyclin dependent kinase is cdc2, cdk2, cdk4, or cdk5.

3.   A method according to claim 1 wherein at least two antisense sequences each complementary to a nucleic a different cyclin or cyclin dependent kinase are administered to the site of vascular lesion formation.

4.   A method according to claim 1 wherein the vascular lesion is associated with cardiovascular angioplasty and the antisense sequence is applied to the angioplasty site to reduce restenosis.

5.   A method according to claim 1 wherein the antisense sequence to cyclin or cyclin dependent kinase is administered in combination with an antisense sequence against proliferating cell nuclear antigen (PCNA).

6.   A method according to claim 1 wherein the antisense sequence is administered in a liposome.

7.   A method according to claim 6 wherein the liposome contains hemagglutinating virus of Japan (HVJ).

8.   A method according to claim 1 wherein the antisense sequence is administered intralumenally, intramurally, or periadventitiously.

9.   A method according to claim 5 wherein the antisense sequences are against cdc2 and PCNA.

10.   A method according to claim 3 wherein the antisense sequences are against cyclin A and cdc2.

11.   A method according to claim 3 wherein the antisense sequences are against cyclin B and cdc2.

12.   A method according to claim 3 wherein the antisense sequences are against cyclin A and cdk2.

13.   A method according to claim 1 wherein said oligomer comprises an alkyl- or aryl-phosphorate oligomer, a phosphotriester oligomer, a carbamate oligomer, a sulfamate oligomer, a morpholino oligomer, a phosphorothioate oligomer, a phosphorodithioate oligomer, a formacetal oligomer, a thioformacetal oligomer or a peptide oligomer.

14.   A method according to claim 13 wherein the antisense oligomer is 12 to 30 nucleotides in length and is stabilized by one or more phosphorothioate linkages.

15.   The method of claim 1 wherein the oligomer is administered in a composite formulated with (N-[1-(2,3-dioleyloxy)propyl]-N,N.N-trimethylammonium chloride as a lipofectin.

16.   A method according to claim 6, wherein said liposome is a cationic, neutral, or anionic liposome.

FIGURE 1
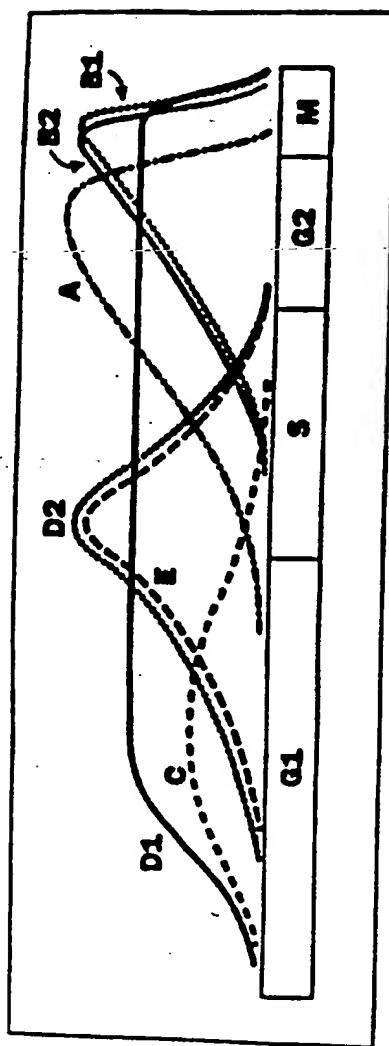
FIGURE 2
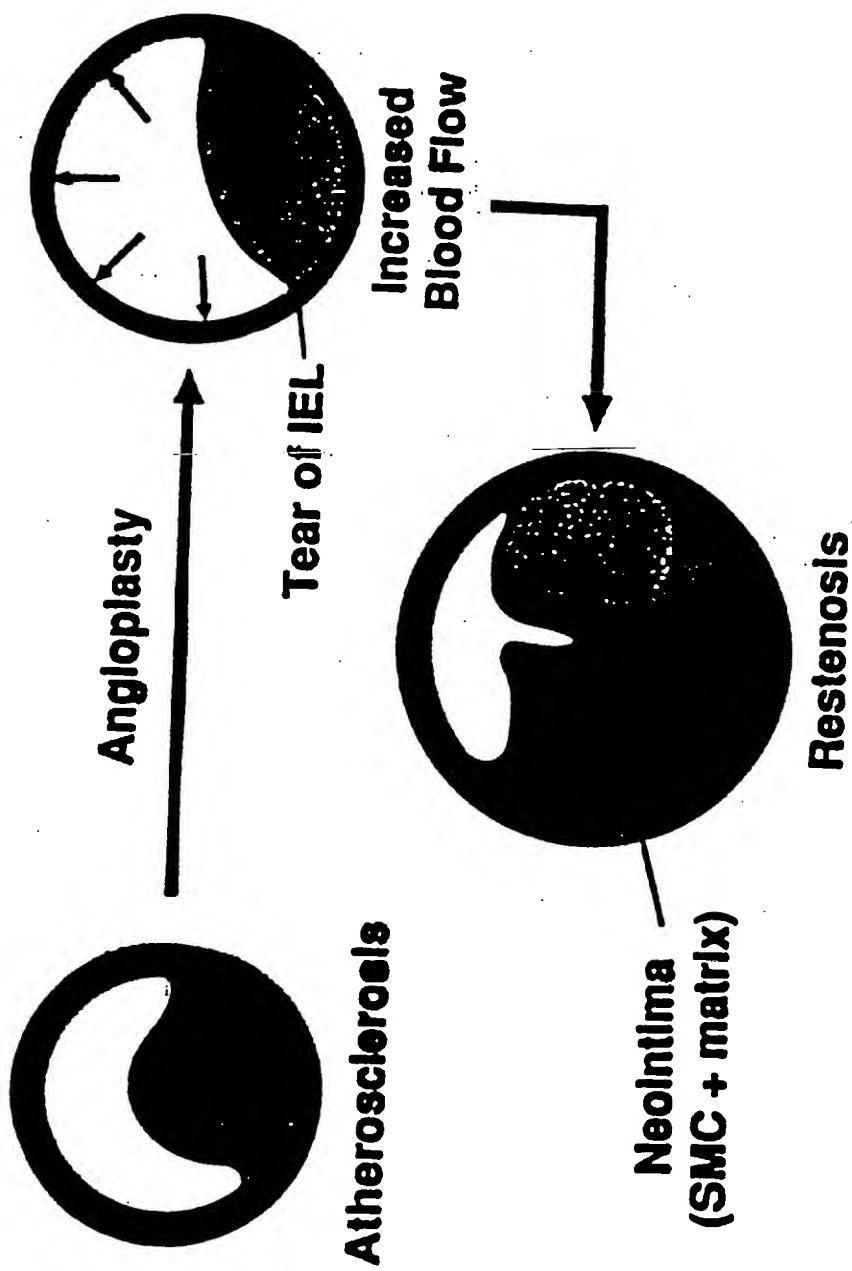
FIGURE 3
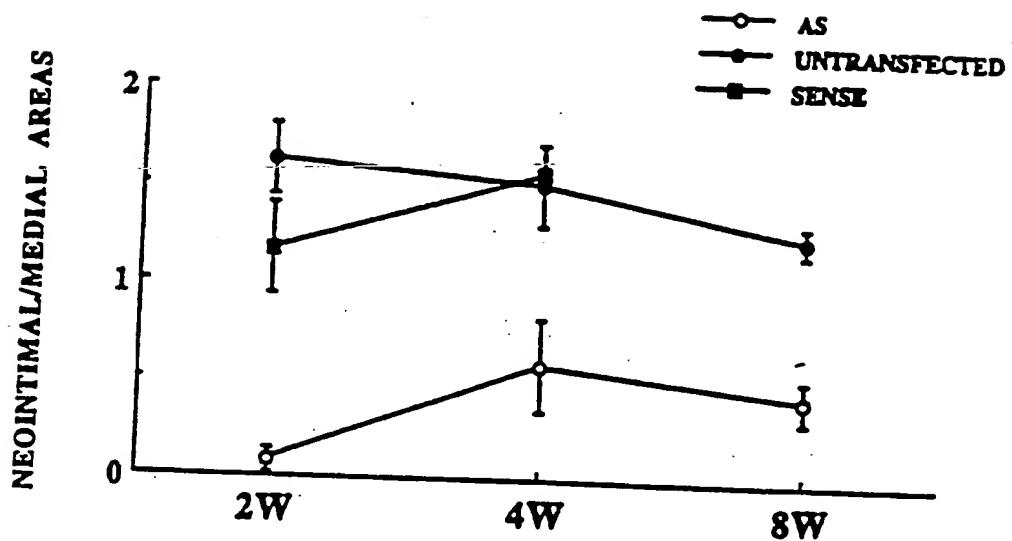
FIGURE   4
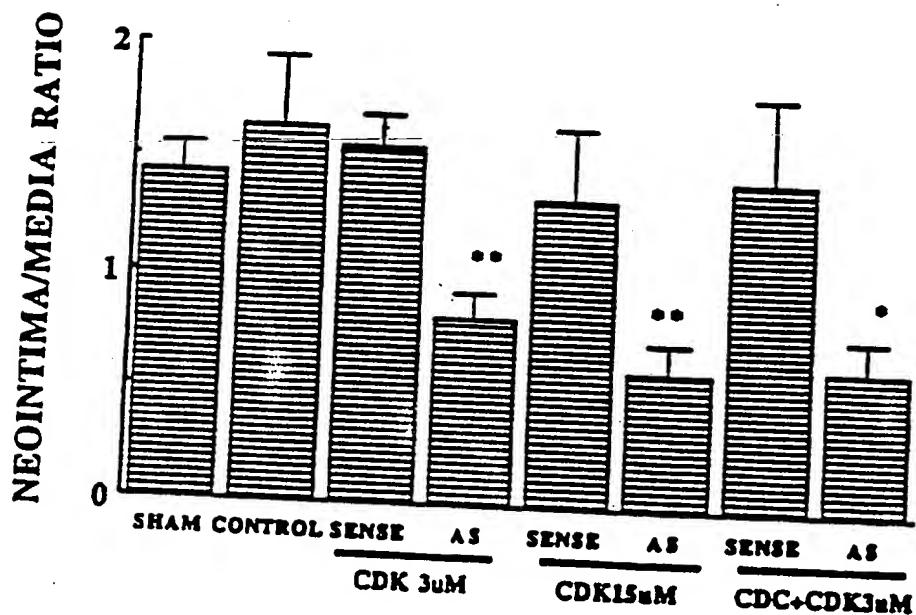
FIGURE 5
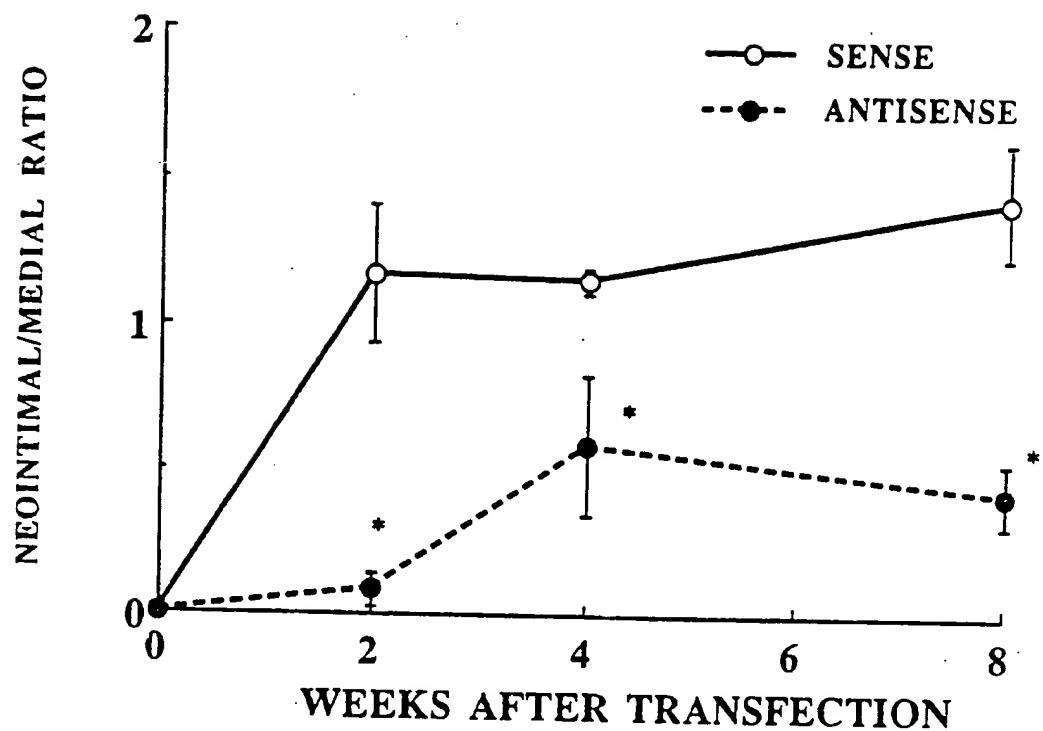
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION F SUBJECT MATTER
IPC 5   C12N15/11   A61K31/70   A61K9/127   C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5   C12N   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CLINICAL RESEARCH, vol.41, no.2, April 1993 page 211A MORISHITA, R. ET AL. 'Novel intraluminal molecular delivery of antisense cdc2 kinase and cyclin A oligonucleotides inhibits neointimal hyperplasia'	1-4,6-8, 10,16
Y	see abstract	5,9, 13-16

Y	MOLECULAR PHARMACOLOGY, vol.41, no.6, June 1992 pages 1023 - 1033 BENNETT, F. ET AL. 'Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides' see abstract	13-16

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| X | Further documents are listed in the continuation of box C. | | Patent family members are listed in annex. |

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

20 September 1994

Date of mailing of the international search report

18. 10. 94

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Authorized officer

Andres, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J.AM.COLL.CARDIOL., vol.19, no.3, 1 March 1992 page 165A PICKERING, J. ET AL. 'Inhibition of proliferation of human vascular smooth muscle cells using antisense oligonucleotides to PCNA' see abstract 759-6	5,9
A	EMBO JOURNAL, vol.10, no.11, 1991, EYNSHAM, OXFORD GB pages 3343 - 3349 VAN LOON, A. ET AL. 'The role of cyclins in the maturation of Platella vulgata oocytes' cited in the application see the whole document	1-3
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.89, no.21, January 1192, WASHINGTON US pages 10415 - 10419 SALA, A. & CALABRETTA, B. 'Regulation of BALB/c 3T3 fibroblast proliferation by B-myb is accompanied by selective activation od cdc2 and cyclin D1 expression' see page 10418, right column - page 10419	1,2
A	SCIENCE, vol.250, 9 November 1990, LANCASTER, PA US pages 805 - 808 FURUKAWA, Y. ET AL. 'cdc2 gene expression at the G1 to S transition in human T lymphocytes' cited in the application see the whole document	1,2
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.90, September 1993, WASHINGTON US pages 8474 - 8478 MORISHITA, R. ET AL. 'Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia' see the whole document	1-9, 13-16

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol.198, no.1, 14 January 1994, DULUTH, MINNESOTA US pages 16 - 24 ABE, J.-I. ET AL. 'Suppression of neointimal smooth muscle cell accumulation in vivo by antisense cdc2 and cdk2 oligonucleotides in rat carotid artery' see the whole document ---	1-4
P,X	THE JOURNAL OF CLINICAL INVESTIGATION, vol.93, no.4, April 1994 pages 1458 - 1464 MORISHITA, R. ET AL. 'Intimal hyperplasia after vascular injury is inhibited by antisense cdk2 kinase oligonucleotides' see the whole document -----	1-4,6-8, 13-16

| Box I | Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet) |

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark: Although claims 1-16 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

| Box II | Observations where unity of invention is lacking (Continuation of item 2 of first sheet) |

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.